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A microanalytical procedure for determination of the base composition of DNA

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A new procedure for the determination of the percentage guanine plus cytosine (% G+C: mol/100 mol) values of microquantities of DNA is described. Its principle is a DNA-polymerase-I-directed nick translation of DNA in the presence of dGTP, dTTP, [^{3}H]dCTP, and [α-^{32}P]dATP. Kinetics experiments indicate that the plateau value is reached in about 20 min of incubation under our experimental conditions. Percentage G+C is obtained from the linear relation $1/(\%\ G+C) = 0.01\ K[^{32}P]/[^3H] + 0.01$, where the ratio of trichloroacetic-acid-precipitable radioactivity is taken into account, the K value being determined for each experiment by using a few reference DNAs of known composition.

This procedure has proven suitable for analysis of plasmidic, viral and cellular DNAs of different base composition (25–75% G + C), shape (linear and circular double-stranded DNA) and size (100–150000 base pairs). Usual methods for % G + C analysis (buoyant density and melting temperature determinations) yield unreliable results in the presence of either modified or unusual bases: the double-labeling procedure is still valid under these conditions. The latter is, therefore, the method of choice for analysis of rare DNA species which are available in very small quantities (it requires amounts of DNA as low as 1 ng, i.e. several order of magnitude lower than those used for chromatographic analysis of DNA hydrolysates). Since the obtention of highly purified DNA is an essential prerequisite for the double-labeling procedure, a method for purification of bacterial DNA is detailed in the present work.

Base composition is an essential parameter for characterization of DNA molecules. Owing to the homologous base pairing A·T and G·C, the guanine-plus-cytosine content, mol/100 mol DNA (% G + C value) is a distinctive trait for double-stranded DNA. The taxonomic significance of G + C values is universally accepted, and determination of base content is widely used in bacterial classification [1]. Tolerance ranges of 10% G + C for a bacterial genus, and of 3% G + C for a species are widely accepted.

Base composition can be determined by quantitative analysis of components (nucleotides, nucleosides, bases) obtained by fractionation of DNA hydrolysates: this procedure is wearisome and requires large amounts of material. Conversely % G + C values can be obtained either from the buoyant density, which is related to the former by a linear relation $\varrho = 1.660 + 0.098\ (G + C)$ [2, 3], or from the thermal denaturation midpoint (t_m), to which the linear relation $t_m = 69.3 + 0.41\ (G + C)$ applies [4–7]. In turn, buoyant density can be obtained from the refractive index of the CsCl solution according to the $\varrho^{25\,°C} = 10.860/n_D^{25\,°C} - 13.4974$ [8].

Buoyant density and t_m determinations are easier and more sensitive procedures, as compared to analysis of DNA hydrolysates. Their main limitation is that the occurrence of either modified or unusual bases (modified bases are the result of postpolymerization processes, whereas unusual bases are synthesized by special metabolic pathways) alters the linear relations linking the three colligative properties of double-stranded DNA. Thus, for example, virus 2C DNA has apparent G + C values of about 80% according to its buoyant density value in CsCl, and of 15% from the established t_m value, whereas base analysis of DNA hydrolysates yields 38% G + C [9–12]: such a discrepancy has been related to a replacement of thymine with hydroxymethyluracil. Similar disagreement among colligative properties has been reported for the DNA containing other unusual bases [13]. Recently a spectrofluorimetric method for bacterial DNA base composition has been described [14]. Unfortunately, till now nothing is known about the applicability of this technique to supercoiled DNA and to DNA with unusual bases.

The present work relates a procedure for analysis of G + C content of double-stranded DNA, which is based on double labelling with one precursor in A·T pair and one precursor in G·C pair. The range of application of this method has been established by analyzing DNA of known composition and of widely different G + C values. More specifically it will be shown that this sort of technique applies equally well to DNA carrying unusual and modified bases, where biophysical methods yield unreliable results. A major advantage of this procedure is the ability to analyze nanogramquantities of DNA: this allows a study of rare DNA species, such as certain plasmids, and the chromosomes of viruses, cell organelles and fastidious organisms.

MATERIALS AND METHODS

Microorganisms and viruses

The following microorganisms were used: *Bacillus subtilis* strains 168/2 (American Type Culture Collection ATCC

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Abbreviations. % G + C, guanine plus cytosine content of DNA (mol/100 mol); t_m, midpoint of optical absorption melting curve.

Enzymes. DNA polI, deoxynucleotidetriphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7); RNase, ribonuclease A (EC 3.1.27.5); lysozyme, mucopeptide N-acetylmuramoylhydrolase (EC 3.2.1.17).

27689) and SB202 (J. Monod Institute, Paris, France). *Clostridium perfringens* (ATCC 3624). *Escherichia coli* strain A19 (*met*$^-$ RNAase$^-$) (Max-Planck-Institute, Berlin, FRG). *Micrococcus lysodeikticus* (syn. *luteus*) (ATCC 4698), *Mycobacterium bovis* BCG (Pasteur Institute, Paris), and *Pseudomonas aeruginosa* (ATCC 10145). Growth was carried out under standard conditions (ATCC catalogue of strains I, XV edition, 1982, Rockville, MD, USA).

Phage T2 was multiplied in *E. coli* strain B (ATCC 11303), and phages 2C and SP01 in *B. subtilis* strain 168/2.

Preparation of DNA

For extraction of bacterial DNA, 10 mg (dry weight) of cells suspended in 500 µl 150 mM NaCl, 10 mM EDTA, 50 mM Tris/HCl pH 7.8 were incubated 14 h at 50°C with 0.5 mg lysozyme, 1 h at 37°C with 0.5 mg pronase and 1 h at 37°C with 1% sodium dodecyl sulfate. Mixtures were repeatedly extracted with chloroform/isoamyl alcohol = 24:1 (v/v) mixture (a procedure similar to that described by Marmur) [15], water-saturated phenol and ether. After incubation with RNase A from bovine pancreas (25 µg/500 µl, 1 h, 37°C), DNA was fractionated by exclusion chromatography on a Sephadex G-50 column previously equilibrated with 4.8 mM sodium phosphate buffer pH 7.8. Fractions eluted in the void volume were purified on hydroxyapatite columns which were successively washed with 0.1 M sodium phosphate buffer pH 7.8 containing 1% (w/v) sodium dodecyl sulfate and 8 M urea, and with 4.8 mM sodium phosphate buffer pH 7.8, and eluted with 480 mM sodium phosphate buffer pH 7.8. Final purification was achieved on Sephadex G-50 columns previously equilibrated with 0.1 mM EDTA, 10 mM Tris·HCl buffer pH 7.8. This procedure proved suitable for extraction and purification of certain kinds of DNA (such as those from mycobacteria), which can hardly be obtained in a pure form with other procedures.

DNA from phages T2 and 2C was obtained by water-saturated phenol and ether extraction, as previously described [16, 17].

Some experiments were performed with purified DNA of commercial sources: thus, the DNA of *Clostridium perfringens*, virus T4 and calf thymus were obtained from Sigma (St Louis, MI, USA), methylated-adenine-free DNA of phage *λ dam*$^-$ (from *E. coli λ* strain C1857) was purchased from Genofit (Geneva, Switzerland), and pBR322 DNA was from Boehringer (Mannheim, FRG).

Polymerase-directed synthesis of labeled DNA

Reaction mixtures for the synthesis of double-labeled DNA had the following composition: 7.5 mM $MgCl_2$, 0.75 mM dithiothreitol, 30 mM Tris/HCl buffer pH 7.4; 5.25 µM dGTP, dTTP and dATP (Boehringer, Mannheim, FRG); 5.25 µM [5-^{3}H]dCTP (28.5 Ci/mmol, New England Nuclear, Dreieich, FRG), 0.004 µM [α-^{32}P]dATP (3200 Ci/mmol, New England Nuclear), and 50 units/ml DNA polymerase I (type 104485 of Boehringer, an endonuclease-containing preparation). Note that the concentrations of the four deoxyribonucleoside triphosphates were similar as were the radioactivity levels of the two labeled precursors.

Polymerization was started by the addition of DNA (5 µl of a solution containing 100 µg DNA/ml added to 10 µl of the reaction mixture), and pursued for 25 min at 20°C. Reaction was stopped by dilution with 10 mM Tris/HCl pH 7.4 10 mM EDTA, and addition of ice-cold 10% trichloroacetic acid containing 1% bovine serum albumin. Samples were filtered through glass-fiber filters (MN85, Macherey-Nagel, Düren, FRG), which were subsequently washed with 5% trichloroacetic acid. Filters were air dried and counted for both ^{3}H and ^{32}P in a scintillation spectrometer: from these values the background values (samples without DNA which were treated in similar way) were subtracted. Correction was made for ^{32}P contamination of the ^{3}H counting channel.

Calculation of the G + C value

In double-stranded DNA the G + C content is related to the ratio of adenine to cytosine:

$$\%\,G + C = \frac{[G] + [C]}{[A] + [T] + [G] + [C]} \times 100 = \frac{100}{\dfrac{[A] + [T]}{[G] + [C]} + 1} = \frac{100}{\dfrac{[A]}{[C]} + 1}.$$

The adenine-to-cytosine ratio was obtained from the trichloroacetic-acid-precipitable radioactivity according to the equation:

$$\%\,G + C = \frac{100}{K\dfrac{[^{32}P]}{[^3H]} + 1}.$$

The K value was determined for each experiment by using several reference DNAs of known composition. It is important to note that this radioactivity ratio indicated the percentages of four nucleotides (mol/100 mol).

By plotting 1/(% G + C) against the ^{32}P/^{3}H ratio, a linear relationship was obtained, which allowed the use of linear regression. Accordingly, the relation $1/(\%\,G + C) = 0.01\,K[^{32}P]/[^3H] + 0.01$ was used, whereby 0.01 K was the slope, and 0.01 the ordinate intercept value.

RESULTS

Determination of the G + C content of different DNA species

The application range of the double-labelling procedure for percentage G + C determination was assessed by submitting to this analytical procedure DNA species widely differing in their composition and structure. The following parameters were considered: (a) source (eucaryotes, procaryotes, plasmids and viruses); (b) conformation (linear, circular and supertwisted molecules); (c) bases (usual, unusual, modified and unmodified nucleotides). Moreover, this study was carried out with 12 reference DNA species, which were previously analyzed in several laboratories by use of both chemical and biophysical techniques [18, 19]. Samples were chosen as to cover the entire range of G + C values, from 25% to 75%.

Data reported in Table 1 indicate a good correspondence (deviation lesser than 1.9%) between the average values found in the literature and those established by the double-labelling procedure. Moreover, with exclusion of *Pseudomonas* DNA the range of variation for five determinations, repeated with the same species of procaryotic and viral DNA, was less than 1.5% G + C. Other conclusions stemming from data in Table 1 include the observation that this procedure applies equally well to DNA species encompassing the whole range of bacterial DNA G + C values (26 – 72% G + C) and of different

Table 1. *Guanine-plus-cytosine values of different DNAs*
% G + C values are averages of five experiments. 'Difference' is that between literature and experimental data

Source of DNA	% G + C		
	experimental	literature	difference
	mol/100 mol		%
M. lysodeikticus	71.8 ± 1.1	71.9	+0.1
P. aeruginosa	64.3 ± 2.5	67.2	+2.9
M. bovis BCG	67.3 ± 0.3	65.0	−2.3
pBR322	51.8 ± 0.8	53.7	+1.9
E. coli	50.8 ± 0.7	50.1	−0.7
λ	50.4 ± 0.8	48.1	−2.3
B. subtilis	44.8 ± 1.3	42.0	−2.8
Calf thymus	40.7 ± 2.5	42.0	+1.3
2C	40.5 ± 1.3	37.9	−2.6
T2	34.0 ± 1.8	34.6	−0.6
T4	32.8 ± 1.0	34.6	+1.8
C. perfringens	27.5 ± 0.7	26.5	−1.0

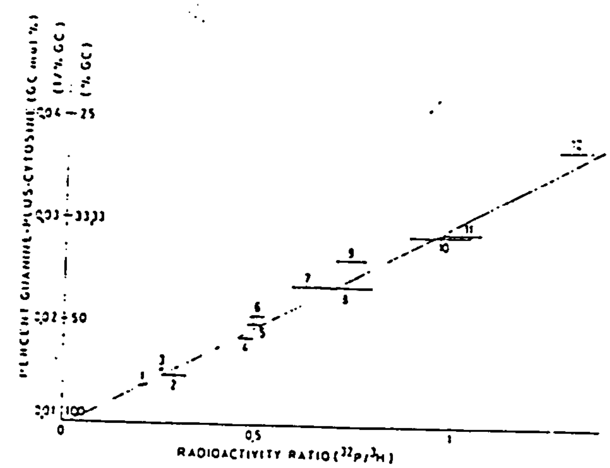

Fig. 1. *Relationship between % G + C value and $^{32}P/^{3}H$ ratio from double-labelling experiments.* The following sources of DNA were considered: *M. lysodeikticus* (1), *P. aeruginosa* (2), *M. bovis* (3), plasmid pBR322 of *E. coli* (4), *E. coli* (5), phage λ of *E. coli* (6), *B. subtilis* (7), calf thymus (8), phage 2C of *B. subtilis* (9), phage T2 of *E. coli* (10), phage T4 of *E. coli* (11) and *C. perfringens* (12). DNA solutions were incubated with DNA polymerase I in a reaction mixture containing the four deoxyribonucleotides with [5-^{3}H]dCTP and [α-^{32}P]dATP as labelled precursors (cf. Materials and Methods). The ^{32}P/^{3}H trichloroacetic-acid-precipitable radioactivity values were plotted against the % G + C literature values. Deviation in five experiments is reported. The corresponding % G + C values were obtained from the relationship 1/(% G + C) = 0.02044 [^{32}P]/[^{3}H] + 0.00967

conformation (circular forms of plasmids as well as linear molecules of cellular and viral DNA). Even more important is the demonstration that the double-labelling method is a suitable tool for analysis of DNA carrying modified bases (bacterial DNA containing methylcytosine and methyladenine), unusual bases (phage 2C containing hydroxymethyluracil in place of thymine) and unusual modified bases (T-even phages, in which cytosine is replaced by hydroxymethylcytosine carrying one or two molecules of glucose).

Linearity of the relationship between the double-labelling determination and the reciprocal value of % C − G appears evident from the graph in Fig. 1. The latter was obtained by

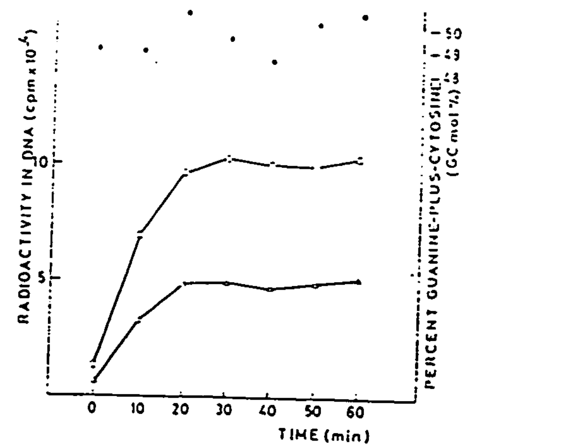

Fig. 2. *Kinetics of incorporation of deoxynucleoside triphosphates into DNA in double-labelling experiments. E. coli* A19 DNA was incubated with DNA polymerase I, dGTP, dTTP, [5-^{3}H]dCTP, and [α-^{32}P]dATP (cf. Materials and Methods). At different times, samples were withdrawn, and the ^{32}P/^{3}H ratio in trichloroacetic-acid-precipitates was determined. Symbols: ^{32}P (○), ^{3}H (△), and % G + C (★)

plotting the [^{32}P]/[^{3}H] values of radioactivity determination against the 1/(% G + C) values in the literature. In this graph the variation range for five determinations is displayed.

Kinetics of DNA double-labelling

Although data in the previous section indicate an unrestricted applicability of the double-labelling technique to analysis of double-stranded DNA irrespective of its origin, conformation and base composition, it is still possible that polymerase I displays a preferential selection of certain domains within a given DNA molecule. If this were the case a careful timing of the length of labelling would be an essential prerequisite for the analytical procedure, since variable results are expected in the course of the polymerisation reaction. This problem was approached by submitting *E. coli* DNA to the double-labelling procedure. Samples of the reaction mixture were withdrawn at different intervals, and the % G + C values were determined.

Data reported in Fig. 2 indicate that incorporation of both ^{32}P and ^{3}H in the course of the polymerization reaction increases for about 20 min and remains at a plateau level for about 1 h. Consequently a standard incubation time of 25 min was chosen for the experiments reported in the present work. The corresponding % G + C values were reasonably close to a straight horizontal line. These results support the inference that faithful % G + C values are expected from the double-labelling procedure, irrespective of the length of the reaction.

Minimum size of DNA

In view of the expected heterogeneity of the genome, the occurrence of a critical size would reasonably be postulated. This problem was tackled by submitting DNA to periods of sonication of different duration. The size of the fragments was evaluated by electrophoresis in the presence of molecular mass standards. Unsonicated (150000 base pairs) and sonicated (down to 100 base pairs) 2C DNA was submitted to double-labelling procedure in the presence of two reference DNAs (*C. perfringens* and *M. lysodeikticus*). As shown in Table 2, DNA segments larger than 100 base pairs, yielded G + C values close to those of the whole genome.

Table 2. *Analysis of DNA of different sizes*
% G + C values are averages of three experiments

Source of DNA	Size (base pairs)	% G + C
		mol/100 mol
2C	150000	37.0 ± 0.2
	405 – 315	36.7 ± 0.6
	405 – 285	36.9 ± 2.2
	240 – 90	37.2 ± 2.3
	< 120	34.1 ± 1.9
C. perfringens	150000 – 1500	26.5 ± 1.2
M. lysodeikticus	100000 – 15000	71.8 ± 2.2

Table 3. *Sensitivity of the procedure for % G + C determination*
% G + C values are averages of five experiments. Labelling efficiency is the ratio (%) of the trichloroacetic-acid-precipitable radioactivity to total radioactivity (background value = 0.5% for ^{32}P and 0.6% for ^{3}H)

Source of DNA	Quantity	% G + C	Efficiency of labelling	
			^{32}P	^{3}H
	ng	mol/100 mol	%	
E. coli	150	49.9 ± 2.3	38.4	54.8
	50	49.7 ± 1.5	21.7	30.6
	15	50.2 ± 1.2	8.5	12.3
	5	48.7 ± 0.6	3.2	4.4
	1.5	50.0 ± 2.4	1.4	2.0
C. perfringens	500	26.6 ± 1.7	10.2	5.4
M. lysodeikticus	500	71.9 ± 1.4	5.1	18.1

Sensitivity of the procedure

The sensitivity of the method was evaluated by determining the G + C value of a wide range of concentrations of DNA. Five quantities of *E. coli* DNA and a control without DNA were analyzed with two reference DNAs (*C. perfringens* and *M. lysodeikticus*). From data in Table 3 it can be inferred that the double-labelling procedure allows the G + C value of nanogram quantities of DNA to be determined with precision.

DISCUSSION

In Materials and Methods a procedure for extraction and purification of bacterial DNA is detailed. This procedure is particularly suitable for isolating the genome of microorganisms of the CMN (*Corynebacterium*, *Mycobacterium* and *Nocardia*) group, which share an unusually tough cell wall, resistant to both mechanical disruption and enzymatic digestion. Moreover, the difficulty of obtaining mycobacterial DNA free of contaminating polysaccharides and proteins has been repeatedly related [20]. In fact, these contaminations interfere with both % G + C determination by biophysical procedures and template function of DNA in duplication and transcription. This justifies the repeated cycles of purification of bacterial DNA by column chromatography. The importance of hydroxyapatite chromatography for DNA purification has been well substantiated [21, 22]. According to our data, purification on hydroxyapatite columns in the presence of detergents and 8 M urea is an essential step for mycobacterial and corynebacterial DNA purification.

The procedure for % G + C determination, which is described in the present work, is based on the polymerization reaction: DNA + deoxynucleoside 5'-triphosphate → DNA (deoxynucleotides $n + 1$) + P_i-P_i, which is catalyzed by DNA polymerase I of *E. coli* and produces a chain growth in the 5' → 3' direction, whereby the last nucleotide is added to the 3'-hydroxyl end of the chain ('tail' type of growth). The template for this sort of reaction ought to be either nicked or gapped double-stranded DNA, since intact duplexes, either linear or circular, are unsuitable as template-primers for polymerase I. For this reason a preparation of DNA polymerase I, containing a well-established quantity of endonucleases, was used in the present work (cf. Materials and Methods). Conversely, the use of pure DNA polymerase I plus a known amount of pancreatic DNase I ought to be considered. DNA polymerase I possesses also a 5' → 3' exonuclease action, supposedly directed toward removal of DNA segments damaged by radiation or altered by mutagens [23]; the latter activity, in conjunction with a primer-dependent polymerization in the 5' → 3' direction, is responsible for the 'nick translation' [24], on which the double-labelling procedure described in the present work is based.

The % G + C value was determined from the labelling ratio of the A·T pairs with a radioactive marker, and of the G·C pairs with another. In the present work [^{32}P]dATP and [^{3}H]dCTP were used, the choice of a very strong and a very weak beta emitter being the most favorable for spectrometric determinations. Although there is no *a priori* discrimination against the use of ^{14}C and ^{35}S-labelled nucleotide precursors, contamination of the ^{3}H channel by either ^{35}S or ^{14}C is expected to be almost twentyfold higher than that by ^{32}P (50% contamination level, instead of 2.5%). Also it must be stressed that the precision of our method requires the use of comparable amounts of radioactivity for the two kinds of precursor. Indeed, a too high ^{32}P radioactivity level produces an exceedingly heavy contamination of the ^{3}H channel and decreases the precision of % C + G measurements.

The occurrence of modified bases in DNA of both procaryotes (25 – 27) and eucaryotes [28] is well established. Modified bases entail the obtention of abnormal buoyant density and t_m data, which lead to erroneous G + C values. Thus, for example, the occurrence of 1% methylated cytosine produces an increase of t_m of 0.18 °C (thus raising the apparent % G + C of 0.32), and a decrease in buoyant density of 0.48 mg cm^{3} (corresponding to a % G + C 0.48 higher) (values calculated after [29]). Unlike these biophysical techniques, the double-labelling method allows faithful % G + C determinations even in the presence of modified and unusual bases (cf. Table 1).

The percentage G + C value can be calculated in several ways from the experimental $^{32}P/^{3}H$ ratios. In the graphic mode, % G + C is obtained by intrapolation using a graph like that in Fig. 1, where the radioactivities ratios are plotted against the 1/(% G + C) values. More precise is a computing approach based on the relation $1/(\% G + C) = a[^{32}P]/[^{3}H] + b$. This consists in calculating a regression line from few reference DNAs of known base composition and covering the whole % G + C range. In practice a couple of extreme standards, such as *C. perfringens* DNA (26.5% G + C) and *M. lysodeikticus* DNA (71.9% G + C) are sufficient for calculating with precision the slope of the line and the value of its intersection with the ordinate axis. Further simplifica-

tion can be obtained by assuming a value of 0.01 for the ordinate intercept, and measuring the % G + C value of a unique reference DNA: the lower the % G + C value of reference DNA, the higher the precision by which the curve slope is measured.

A main objection to the procedure outlined in this paper is that polymerase I might nick-translate preferentially DNA segments of a given composition. Since, however, this procedure was proven to apply equally well to DNA of quite different G + C content (Fig. 1), it can be inferred that the possible heterogeneity of the genome does not stand out, when analysis is carried out on sufficiently long DNA segments. This finding agrees with the report that DNA polymerase I has no preferential duplication sites [24] (which is also applicable, our data show, to DNA with unusual bases). Analysis of small DNA segments of different composition might be able to disclose a microheterogeneity of DNA; however, data in Table 2 indicate that the limit of applicability of our procedure is about 120 base pairs.

The double-labelling procedure for % G + C determination presents the following advantages with respect to the available methods: (a) it is much shorter (buoyant density determinations by centrifugation in CsCl gradients require days); (b) it is relatively inexpensive (compared to the costly equipment required for t_m and buoyant density measurements); (c) it is not limited by the presence of modified and unusual bases nor by the occurrence of base-linked hexoses (as are t_m and buoyant density determinations); and (d) it requires nanogram quantities of DNA (microgram amounts of DNA are required for chromatographic analysis of DNA hydrolysates). Since % G + C determination by biophysical procedures is always uncertain without parallel chemical analysis (which requires large amounts of material), the double-labelling procedure outlined in the present paper is the method of choice when very small amounts of DNA are available.

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